



FLUOROMETRY AND FLUOROMETRIC DEVICE AND SUBSTRATE
SUITABLE THEREFOR

BACKGROUND OF THE INVENTION

5 Field of the Invention

The present invention relates to a method and a device for measuring fluorescence of samples, in particular, to method for measuring fluorescence of samples on a surface of a disk-shaped substrate. The present invention also relates to a substrate suitable for the fluorometry.

Related Background Art

The use of fluorescence is indispensable for the measurements for a very slight amount of substances such as samples taken from organisms. There are some other means of measuring for a very slight amount of substances, such as radioisotope and nuclear magnetic resonance, however, the fluorometry has the widest applications in terms of sensitivity, wide variety of labeling substances applicable, easiness of handling, usability for general-purpose, improvement in light detection sensitivity.

The fluorometry is applied not only to staining cells and tissues, which is for making the same visible, but also to quantifying substances since it is capable of making measurements quantitatively. Particularly in the detection of a very slight amount

of substances, it can form a detection system of high sensitivity and specificity in combination with specific reactions, such as antigen-antibody reaction with biomolecules and hybridization with DNAs having
5 specific base sequences.

One of the requirements in the measurement of fluorescence is the separation of excitation light. This means that fluorescence alone has to be measured by making some devices for preventing excitation light
10 from approaching the light detecting portion.

For example, in an ordinary erection type fluorescence microscope, a dichroic mirror and a filter are mounted thereon so as to prevent the excitation light reflected or scattered by samples from
15 approaching the light detecting portion. In a device for measuring the lifetime of fluorescence, the excitation light is prevented from approaching the light detecting portion by the use of a pulse light emission source at the time of measuring fluorescence.
20 And in a spectrofluorometer, the excitation light is made to approach the light detecting portion as rarely as possible by allowing the excitation light and the optical axis of the light detecting portion to be perpendicular to each other.

25 The use of the above methods enables performing the measurement of fluorescence while preventing the majority of the excitation light from approaching the

light detecting portion. However, it goes without saying that even the above devices cannot prevent 100 % of the excitation light from approaching the light detecting portion and, in actuality, some of the excitation light leaks to the light detecting portion, though it is very slight compared with the intensity of the excitation light.

SUMMARY OF THE INVENTION

When the amount of the samples is relatively large and fluorescence is easy to observe, or when the fluorescent substance contains a lot of fluorescent dye with a sufficient intensity, the approach of a slight amount of excitation light to the light detecting portion (leaky light) is not a problem and does not affect the measurement of fluorescence. This is because the intensity of the fluorescence emitted is overwhelmingly high compared with the leaky light and the leaky light does not affect the situation as a whole.

However, when measuring a slight amount of faint fluorescence, the leaky light can sometimes affect the measurement seriously. According to the situation, the amount of the leaky light may be larger than that of the fluorescence emitted.

There are cases where a band-pass filter is inserted in front of the light detecting portion, as

the preventive measures; however, since the
fluorescence subjected to measurement is faint, the
insertion of such a filter sometimes result in
insufficient amount of fluorescence subjected to
5 measurement.

Further in many fluorochromes, their fluorescent
wavelength is often close to that of the excitation
light, and there may be cases where a part of the short
wavelength region of the fluorescence and a part of the
10 long wavelength region of the excitation light overlap
each other; thus, the above two types of light are hard
to separate clearly in principle.

In recent years, the sensitivity of cameras has
been substantially increased and measurement of light
even at a photon counting level has been made possible.
15 Therefore, if the excitation light can be removed more
efficiently and the measurement of faint fluorescence
is made possible, the merit of the application of
fluorometry is more increased.

Accordingly the object of the present invention is
20 to overcome the above-described problems, in other
words, to remove the excitation light from the light
detecting portion more effectively.

Specifically, according to one aspect of the
25 present invention, there is provided a fluorometry for
measuring fluorescence emitted from samples on a
measuring surface of a substrate by illuminating the

samples with excitation light, characterized in that

an excitation light illumination portion and a
light detecting portion are placed in such a manner as
to make it possible to prevent the excitation light
from approaching the light detecting portion where
measurement of the fluorescence is performed,

the fluorescence emitted from the samples is
measured by relatively moving the samples on the
measuring surface of the substrate from the excitation
light illumination portion to the light detecting
portion after illuminating the samples with the
excitation light.

Preferably, the method of the present invention is
characterized in that a circular orbit is formed on the
measuring surface of the substrate by relatively moving
the samples.

Preferably, the method of the present invention is
characterized in that the circular orbit is formed
while forming a rotational plane of the measuring
surface by rotating the substrate around the axis
perpendicular to the measuring surface thereof and the
samples are moved from the excitation light
illumination portion to the light detecting portion
relative to the rotational plane of the measuring
surface.

Or preferably, the method of the present invention
is characterized in that the circular orbit of the

measuring area is formed by allowing the excitation light illumination portion and the light detecting portion to perform a rotational movement.

5 Preferably, the method of the present invention is characterized in that the samples are liquid filled in cells formed on the substrate or are substances fixed on, adsorbed onto, or trapped in the substrate, and that the samples are fixed in the probes arranged on the substrate.

10 Preferably, the method of the present invention is characterized in that the probes and the sample are DNA, protein or peptide nucleic acid (PNA).

15 Preferably, the method of the present invention is characterized in that the spacing between the excitation light illumination position and the light detecting portion is variable or the moving speed of the sample is variable, and that the period from the illumination of excitation light to the detection of fluorescence is properly adjustable by varying the moving speed.

20 Preferably, the method of the present invention is characterized in that the samples on the measuring surface of the substrate are arranged on more than one circles or on the arcs thereof which are concentric with the central axis of the substrate and are different in radius from each other, and that the samples at the same distance from the central axis have

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the same or similar attributes and form a group which can be discriminated from the other groups of samples.

According to another aspect of the present invention there is provided a substrate having samples, which are subjected to fluorescence measurement, on its measuring surface, characterized in that the samples are arranged on more than one circles or the arcs thereof which are concentric with the central axis of the substrate and are different in radius from each other, and that the samples at the same distance from the central axis have the same or similar attributes and form a group which can be discriminated from the other groups of samples.

According to further aspect of the present invention there is provided a fluorometric device which includes an excitation light illumination portion where samples on the measuring surface of a substrate are illuminated with excitation light and a light detecting portion where the measurement of the fluorescence emitted from the samples is performed, characterized in that

the excitation light illumination portion and the light detecting portion are placed in such a manner as to make it possible to prevent the excitation light from approaching the light detecting portion,

a means is provided for relatively moving the samples on the measuring surface of the substrate from

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the excitation light illumination portion to the light detecting portion,

the means for relatively moving the samples being preferably such that it moves the substrate having the samples on the measuring surface relative to the excitation light illumination portion and the light detecting portion while allowing the same to form a circular orbit.

10 BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a view of a fluorometric device in accordance with the present invention;

FIG. 2 is an enlarged view of the stage portion;

15 FIG. 3 is a view showing the positional relation between the excitation light illumination portion and the light detecting portion;

FIG. 4 is a graphical representation of the fluorescent spectrum of a sample obtained by a fluorometric device; and

20 FIG. 5 is a view of a substrate on which 4 types of probes are arranged in such a manner as to form concentric circles.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

25 The fluorometry and fluorometric device in accordance with the present invention are characterized in that the light detecting portion is placed in such a

position that excitation light does not enter it and a mechanism is provided which prevents the excitation light from approaching the light detecting portion by moving samples from the excitation light illumination portion to the light detecting portion after illuminating the samples with the excitation light.

The optical axis of the excitation light and that of the light detecting portion are completely different from each other, thereby the excitation light can be inhibited from approaching the light detecting portion in principle. However, in view of the influence of excitation light propagation and scattering in the glass due to its reflection within the substrate, the inhibition of the excitation light from approaching the light detecting portion can be ensured by taking the following measures:

a) using black glass for the substrate instead of glass;

b) covering both the excitation light illumination portion and the light detecting portion so as to block off the scattered light spatially; and

c) increasing the flatness of the substrate surface and illuminating the substrate with the excitation light perpendicular thereto so as to decrease the light scattering on the surface of the substrate.

It goes without saying that desirably the movement

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measurements can be made and their results can be integrated.

When the samples are hard to rotate, they may be moved linearly in a fixed direction. In such a case, the excitation light illumination portion and the light detecting portion are properly spaced and the samples having been illuminated with the excitation light are moved in such a manner as to reach the light detecting portion while they are emitting fluorescence, just like the cases where the samples are rotated.

In many cases, it is easier to move the samples than to move both the excitation light illumination portion and the light detecting portion, in terms of the device's structure; however, when the samples are hard to move, the excitation light illumination portion and the light detecting portion may be moved relative to the samples.

More than one excitation light illumination portions and light detecting portions can be placed. For example, the samples can be subjected to measurements at more than one different spots under different conditions after being illuminated with excitation light at one spot. Alternatively, in view of the efficiency in measuring time, measurements can be made at more than one different spots in parallel.

The period of time from the excitation light illumination to the samples' passing through the light

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detecting portion depends on the spacing between the
excitation light illumination portion and the light
detecting portion as well as the moving speed of the
samples. Because the lifetime of fluorescence varies
5 depending on the kinds of, for example, dyes emitting
fluorescence, the device is desirably such that the
spacing between the excitation light illumination
portion and the light detecting portion as well as the
moving speed of the samples are variable.

10 The fluorometry in accordance with the present
invention is applicable to the substance detection
using a solid-phase substrate, to which attention has
been being paid in recent years. When making
measurements for samples using fluorescence after
15 reacting the samples with a substrate, in particular, a
solid-phase substrate on which probes such as DNA and
proteins are provided, the measurements have to be made
for a very slight amount of fluorescent substance on
the substrate. And in order to make high-sensitivity
20 measurements, efficient removal of excitation light is
a key factor. The fluorometry in accordance with the
present invention enables the improvement in removal of
excitation light.

When utilizing the fluorometric device in
25 accordance with the present invention for the micro-
measurements using the substrate described above,
measurements with more than one types of probes are

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made possibly only by allowing the light detecting portion on the substrate to take the form of concentric circulars, that is, by allowing each of the probes to have a pattern of a circle with its center at the rotational center of the substrate and using a substrate on which the probes are arranged in the form of concentric circles with different radii. In this case, measurements are made while adjusting the light detecting portion (usually objective lens) to the rotating substrate, and measurements for the sample corresponding to more than one probes can be performed by varying the distance from the rotational center to each probe.

Examples

(Example 1)

(1) Preparation of Substrate

A silica glass substrate 30 mm in diameter and 0.5 mm in thickness was prepared. In order to allow the substrate to rotate, the circular portion of the substrate within 10 mm from its center was designed in such a manner as to make it possible to transmit the rotational drive thereto.

The silica glass substrate was water-washed lightly, subjected to ultrasonic cleaning for 20 minutes while being immersed in an exclusive substrate cleaning solution, and left stand one whole day and night. Then the substrate was taken from the cleaning

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5 solution, washed with water and deionized water so as to take the cleaning solution away therefrom, and immersed in 1M NaOH aqueous solution preheated to 60°C for 20 minutes. The substrate was taken from the NaOH aqueous solution, again washed with water and deionized water so as to take the NaOH aqueous solution away therefrom, and subjected to ultrasonic cleaning in deionized water for 20 minutes.

10 Then the substrate was immersed in a silane coupling agent (manufactured by Shin-Etsu Chemical Co., Ltd., brand name: KBM 603), which was previously dissolved in water to be 1 % aqueous solution and subjected to hydrolysis for about one hour, for one hour. The substrate was washed lightly with deionized water, dried by blowing the water droplets remaining on the surface away with nitrogen gas, and baked in an oven at 120°C for 2 hours. Amino groups were introduced to the glass surface by attaching the silane coupling agent to the same.

20 (2) Dye Attachment

Then 2 ml of a solution of eosin-5-isothiocyanate, a protein labeling dye manufactured by Funakoshi Co., Ltd., in 1M NaCl/50 mM sodium phosphate buffer solution (pH 7.0) (50 µM concentration) was prepared, and the solution was reacted with the substrate for 10 hours in a hybridization package. After the completion of the reaction, the substrate was washed with 1M NaCl/50 mM

sodium phosphate buffer solution (pH 7.0) to take the unreacted eosin-5-isothiocyanate away therefrom, and a substrate 1 with eosin attached thereto was obtained.

(3) Device Construction

5 A measuring device shown in FIG. 1 was prepared by remodeling the stage portion of a fluorescence microscope manufactured by Nikon Corporation. FIG. 2 is an enlarged view of the stage portion and its vicinities. An excitation light illumination portion 10 13a and an objective lens 7 of the fluorescence microscope, as a light detecting portion 15, are placed on the opposite sides relative to the central axis of the disk, and on the central portion of the disk a rotational drive device was placed for rotating the 15 substrate. Although the distance from the central axis to the excitation light illumination portion and that from the central axis to the light detecting portion 15 were variable, both the portions were fixed on the opposite sides relative to the central axis at a 20 distance 12.5 mm from the central axis. In other words, both the excitation light illumination portion and the light detecting portion 15 were placed in such a manner that, when the disk was turned 180°, the portion illuminated with excitation light was moved to 25 the light detecting portion 15 (see FIG. 3).

The measuring device was designed in such a manner that the light obtained from the objective lens 7, as

the light detecting portion 15, was sent to a
fluorometric device 12 via an optical fiber 11
extending from the microscope 5, so as to be analyzed
there. This fluorometric device 12 is capable of
5 integrating data, and when the fluorescence obtained is
very faint, its sensitivity can be increased by
integrating data.

(4) Substrate Measurement

10 The previously prepared substrate 1 with eosin
attached thereto was fixed on the remodeled stage. The
rotation speed of the disk was set at 30000 rpm and the
excitation light at 522 nm.

15 Measurements were made while illuminating the
samples with excitation light and continued for 10
seconds until sufficient sensitivity was obtained, and
the fluorescence detected from the substrate was
integrated. As a result, the spectrum shown FIG. 4 was
obtained.

20 There was no appreciable peak around the
wavelength of 522 nm, which was the wavelength of the
excitation light, and the fluorescence derived solely
from eosin was measured.

25 Thus, the fluorometry in accordance with the
present invention enabled the measurement of the
fluorescence of the samples without excitation light.

(Example 2)

(1) Preparation of Substrate

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5 A silica glass substrate 30 mm in diameter and 1 mm in thickness was prepared. In order to allow the substrate to rotate, the circular portion of the substrate within 10 mm from its center was designed in such a manner as to make it possible to transmit the rotational drive thereto.

10 The silica glass substrate was water-washed lightly, subjected to ultrasonic cleaning for 20 minutes while being immersed in an exclusive substrate cleaning solution, and left stand one whole day and night. Then the substrate was taken from the cleaning solution, washed with water and deionized water so as to take the cleaning solution away therefrom, and immersed in 1 M NaOH aqueous solution preheated to 60°C
15 for 20 minutes. The substrate was taken from the NaOH aqueous solution, again washed with water and deionized water so as to take the NaOH aqueous solution away therefrom, and subjected to ultrasonic cleaning in deionized water for 20 minutes.

20 Then the substrate was immersed in a silane coupling agent (manufactured by Shin-Etsu Chemical Co., Ltd., brand name: KBM 603), which was previously dissolved in water at a final concentration of 1 % and subjected to hydrolysis for about one hour, for one
25 hour. The substrate was washed lightly with deionized water, dried by blowing the water droplets remaining on the surface away with nitrogen gas, and baked in an

oven at 120°C for 2 hours. Amino groups were introduced to the glass surface by attaching the silane coupling agent to the same.

Then a crosslinking agent, EMCS (N-(6-Maleimidocaproyloxy)succinimide), manufactured by Dojindo Laboratories was dissolved in a mixed solvent (ethanol : DMSO = 1 : 1) in the proportion of 3 mg to 10 ml. The substrate having been baked previously was immersed in the obtained EMCS solution and left for 2 hours. After taking the substrate from the EMCS solution and washing the same lightly with the same mixed solvent as above, the droplets remaining on the surface of the substrate was subjected to substitution into ethanol, and the substrate was dried by blowing the droplets away with nitrogen gas. Thus a substrate (EMCS-treated substrate) was obtained the entire surface (the surface of both sides) of which EMCS was attached to. EMCS contains a succinimide group and a maleimido group; and since the succinimide group binds to the amino group on the surface of the substrate, the surface of the substrate had the maleimido group having been introduced thereon.

(2) DNA Attachment

Modified 18-mer DNA (probe) with a thiol group (SH group) attached to one terminal thereof was synthesized by BEX at the request of the applicants of the present invention. The SH group was attached to 5' terminal

and the DNA base sequence was as follows:

5'HS-ACTGGCCGTCGTTTACA³'

(SEQ ID No. 1)

5 The above DNA was dissolved in water at a final
concentration of 30 μ M. Two ml of the obtained DNA
aqueous solution was sealed into a hybridization
package together with the previously prepared EMCS-
treated substrate, so as to subject them to
hybridization reaction for 2 hours. After the
10 completion of the reaction, the substrate was washed
with 1M NaCl/50 mM sodium phosphate buffer solution (pH
7.0) so as to completely wash the DNA solution away
from the glass surface. Then the substrate was
immersed in 2 % bovine serum albumin aqueous solution,
15 left for 2 hours, and subjected to blocking reaction.
After the blocking reaction, the substrate was again
washed with 1M NaCl/50 mM sodium phosphate buffer
solution (pH 7.0), so as to obtain the substrate to
which DNA was attached.

20 (3) Synthesis of EOSIN-Labeled DNA

Amino-linked 18-mer DNA having a sequence
complementary to that of the DNA attached to the
substrate was synthesized by BEX at the request of the
applicants of the present invention. As the amino
25 group, used was a commonly used 5'-terminal-
hexamethylene type amino linker.

5'-terminal-eosin-labeled DNA was obtained by

5 (4) Hybridization

After the reaction, the substrate was washed with 1M NaCl/50 mM sodium phosphate buffer solution (pH 7.0), so as to obtain the substrate 1, as the subject of measurements.

Measurements were made for the obtained substrate 1 with the measuring device used in the example 1. The rotation speed of the disk was set at 30000 rpm and the excitation light at 522 nm, like in the example 1.

Like in the example 1, there was no appreciable

peak around the wavelength of 522 nm, and the
fluorescence derived solely from eosin was measured.
Thus, the fluorometry in accordance with the present
invention enabled the measurement of the fluorescence
5 of the samples without excitation light.

(Example 3)

(1) Preparation of Substrate

An EMCS-treated substrate, the entire surface of
which EMCS was attached to, 30 mm in diameter was
10 obtained in the same manner as in the example 2 (1).

(2) DNA Attachment

Modified 18-mer DNAs (probes) with a thiol group
(SH group) attached to one terminal thereof were
synthesized by BEX at the request of the applicants of
15 this invention. The SH group was attached to 5'
terminal and the DNA base sequence was as follows:

No. 1: 5'HS-ACTGGCCGTCGTTTTACA^{3'}

(SEQ ID No. 1)

No. 2: 5'HS-ACTGGCCGTTGTTTTACA^{3'}

20 (SEQ ID No. 2)

No. 3: 5'HS-ACTGGCCGCTTTTTTTACA^{3'}

(SEQ ID No. 3)

No. 4: 5'HS-ACTGGCATCTTGTTTTACA^{3'}

(SEQ ID No. 4)

25 The above DNAs were dissolved in SG Clear (aqueous
solution containing 7.5 % of glycerol, 7.5 % of urea,
7.5 % of thiodiglycol and 1 % of acetylenol EH), which

is a solvent for use in thermal jet printers, and
adjusted at a final concentration of 8 μ M. And a
cartridge for use in thermal jet printers is filled
with the DNA solution. The four types of probes 16 are
5 arranged in the pattern shown in FIG. 5. Specifically,
the probe No. 1 was arranged on the circumference with
a radius of 14 mm, the probe No. 2 on the circumference
with a radius of 13 mm, the probe No. 3 on the
circumference with a radius of 12 mm and the probe No.
10 4 on the circumference with a radius of 11 mm in such a
manner that all of them formed concentric circles (the
probes are arranged in such a manner as to form
concentric circles spaced at intervals of 1 mm in the
order, No. 1, No. 2, No. 3 and No. 4 from the outside
15 inward).

Then the substrate with the DNA solution placed
thereon was left in a humid chamber for 30 minutes to
react the substrate with DNA.

The thermal jet printer used was a remodeled type
20 of thermal jet printer C-600, which is manufactured by
Canon Inc. and capable of doing lithographic printing.

After the completion of the reaction, the
substrate was washed with 1M NaCl/50 mM sodium
phosphate buffer solution (pH 7.0), so as to wash the
25 DNA solution away from the glass surface completely.
Then the substrate was immersed in 2 % bovine serum
albumin aqueous solution, left for 2 hours, and

subjected to blocking reaction. After the blocking reaction, the substrate was again washed with 1M NaCl/50 mM sodium phosphate buffer solution (pH 7.0), so as to obtain the substrate to which 4 types of DNAs were attached in the form of concentric circles.

(3) Synthesis of EOSIN-Labeled DNA

Amino-linked 18-mer DNA having a sequence complementary to that of the DNA attached to the substrate was synthesized by BEX at the request of the applicants of the present invention. As the amino group, used was a commonly used 5'-terminal-hexamethylene type amino linker.

5'-terminal-eosin-labeled DNA was obtained by reacting the amino-linked DNA with eosin-5-isothiocyanate, a protein-labeling dye manufactured by Funakoshi Co., Ltd. Labeling reaction was carried out by conventional procedure.

(4) Hybridization

Hybridization was carried out in the same manner as in the example 2 (4).

(5) Substrate Measurement

Measurements were made for the obtained substrate 1 with the measuring device used in the example 1. The rotation speed of the disk was set at 30000 rpm and the excitation light at 522 nm, like in the example 1.

The fluorescence spectrum at the portion to which probes 16 were attached was measured for each probe in

order while allowing the disk to rotate and properly
setting the positions of the excitation light
illumination portion 13a and the objective lens 7. As
a result, the fluorescence spectrum could be measured
5 for 3 probes other than probe No. 4.

The measurements at 560 nm, where the intensity of
fluorescence was the maximum, were as follows: 2300 for
the probe No. 1, 1400 for the probe No. 2, and 1000 for
the probe No. 3.

10 The results show that the more the base sequence
of a sample is complementary to that of the probe, the
more the fluorescence is intensive. And it is apparent
from the results that the analysis of base sequences of
nucleic acid can be performed with a fluorometric
15 device in accordance with the present invention and a
substrate on which more than one probes are arranged in
the form of concentric circles.

The fluorometry in accordance with the present
invention has the advantage of being capable of
20 removing excitation light without a complicated device
construction. It provides complete removal of
excitation light, since its light detecting portion is
placed in such a position that excitation light cannot
approach.

25 The forms of providing samples for the
fluorometric device in accordance with the present
invention include a disk-shaped substrate. And in the

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use of such a disk-shaped substrate, measurements for
the samples using more than one probes can be easily
performed by arranging those probes on the substrate in
the form of concentric circles with their centers at
5 the rotational axis and with radii different from each
other.

(SEQUENCE LISTING)

SEQ ID No.: 1
Length: 18
10 Type: Nucleic Acid
Strandedness: Single Stranded
Topology: Linear
Molecule type: Other Nucleic Acid, Synthetic DNA
Sequence description:
15 ACTGGCCGTC GTTTACA 18

SEQ ID No.: 2
Length: 18
Type: Nucleic Acid
20 Strandedness: Single Stranded
Topology: Linear
Molecule type: Other Nucleic Acid, Synthetic DNA
Sequence description:
ACTGGCCGTT GTTTACA 18

25

SEQ ID No.: 3
Length: 18

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Type: Nucleic Acid

Strandedness: Single Stranded

Topology: Linear

Molecule type: Other Nucleic Acid, Synthetic DNA

5 Sequence description:

ACTGGCCGCT TTTTACA 18

SEQ ID No.: 4

Length: 18

10 Type: Nucleic Acid

Strandedness: Single Stranded

Topology: Linear

Molecule type: Other Nucleic Acid, Synthetic DNA

Sequence description:

15 ACTGGCATCT TGTTTACA 18

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